Li R, Jin Z, Gao L, Liu P, Yang Z, Zhang D. 2016. Effective protein inhibition in intact mouse oocytes through peptide nanoparticle-mediated antibody transfection. PeerJ 4:e1849
https://doi.org/10.7717/peerj.1849

Effective protein inhibition in intact mouse oocytes through peptide nanoparticle-mediated antibody transfection

Ruichao Li, Zhen Jin, Leilei Gao, Peng Liu, Zhixia Yang, Dong Zhang

Female meiosis is a fundamental area of study in reproductive medicine, and the mouse oocyte model of in vitro maturation (IVM) to study female meiosis is the most widely used. To investigate the probable role(s) of an unknown protein in female meiosis, the method traditionally used involves microinjecting a specific antibody into mouse oocytes. Recently, in studies on somatic cells, peptide nanoparticle-mediated antibody transfection has become a popular tool because of its high efficiency, low toxicity, good stability, and strong serum compatibility. However, till now, no researchers have tried using this technique on mouse oocytes because the zona pellucida surrounding the oocyte membrane (vitelline membrane) is usually thought or proved to be a tough barrier to macromolecules, such as antibodies and proteins. Therefore, we attempted to introduce an antibody into mouse oocytes using a peptide nanoparticle. Here we show for the first time that with our optimized method, an antibody can be effectively delivered into mouse oocytes and inhibit its target protein with high specificity. We obtained significant results using small GTPase Arl2 as a test subject protein. We propose peptide nanoparticle-mediated antibody transfection to be a superior alternative to antibody microinjection for preliminary functional studies of unknown proteins in mouse oocytes.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1566v1 | CC-BY 4.0 Open Access | rec: 5 Dec 2015, publ: 5 Dec 2015

Effective protein inhibition in mouse oocytes through peptide nanoparticle-mediated antibody transfection

Ruichao Li[1], Zhen Jin[1], Leilei Gao[1], Peng Liu[1], ZhixiaYang[1,2], Dong Zhang[1,2]

[1]State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 210029, China;

[2]Correspondence to:

dong.ray.zhang@njmu.edu.cn and yang_zhixia@sina.com

ABSTRACT

Female meiosis is a fundamental area of study in reproductive medicine, and the mouse oocyte model of in vitro maturation (IVM) to study female meiosis is the most widely used. To investigate the probable role(s) of an unknown protein in female meiosis, the method traditionally used involves microinjecting a specific antibody into mouse oocytes. Recently, in studies on somatic cells, peptide nanoparticle-mediated antibody transfection has become a popular tool because of its high efficiency, low toxicity, good stability, and strong serum compatibility. However, till now, no researchers have tried using this technique on mouse oocytes because the zona pellucida surrounding the oocyte membrane (vitelline membrane) is usually thought or proved to be a tough barrier to macromolecules, such as antibodies and proteins. Therefore, we attempted to introduce an antibody into mouse oocytes using a peptide nanoparticle. Here we show for the first time that with our optimized method, an antibody can be effectively delivered into mouse oocytes and inhibit its target protein with high specificity. We obtained significant results using small GTPase Arl2 as a test subject protein. We propose peptide nanoparticle-mediated antibody transfection to be a superior alternative to antibody microinjection for preliminary functional studies of unknown proteins in mouse oocytes.

INTRODUCION

Female meiosis studies in mammals are very relevant to the reproductive health of female humans and have the potential to benefit both basic reproductive medicine and clinical diagnosis

and therapies for human reproductive disorders. However, female mammalian meiosis studies are progressing much more slowly than general mammalian mitosis studies (publication in PubMed, 14.1% of general). One of the biggest hurdles is that to know down a gene or inhibit a protein, which is usually the first step of the study, researchers have to perform microinjection to introduce siRNA or a specific antibody into oocytes. Compared with transfection, the disadvantages of microinjection include possible mechanical damage, difficulty with dose control, and a substantially longer time requirement.

Protein inhibition by a specific antibody is one of the most powerful tools in cell biological studies. Compared with siRNA-mediated gene silencing, inhibition by an antibody is more specific because the antigen–antibody association possesses the highest specificity. In addition, protein inhibition can usually take effect much faster than siRNA silencing. Traditionally, microinjecting the corresponding antibody into cells was the only way to inhibit a specific subject protein (Mehlmann & Jones & Jaffe, 2002; Yin et al., 2006; Wang et al., 2008). In recent years, antibody delivery through peptide nanoparticle-mediated transfection has emerged as a superior alternative because of its high efficiency, low toxicity, good stability, and strong serum compatibility (Kondo et al., 2008; Aoshiba & Yokohori & Nagai, 2003; Morris et al., 1999; Morris et al., 2001). Another key advantage is that there are diverse commercial antibodies available against a large portion of functionally unknown proteins in the human and mouse proteomes (Shirai et al., 2014). However, in oocytes, whether peptide nanoparticle-mediated antibody transfection can effectively deliver antibodies into oocytes has never been tested.

Our lab has been attempting to deliver antibodies into mouse oocytes without the use of microinjection. After screening several peptide nanoparticle transfection reagents and testing many protocols, we have identified the most appropriate one (Aoshiba & Yokohori & Nagai, 2003; Morris et al., 1999; Morris et al., 2001) and successfully developed a feasible standardized approach for use with mouse oocytes.

MATERIALS AND METHODS

General chemicals & reagents and animals

Chemicals & reagents were obtained from Sigma unless otherwise stated. ICR mice used in this study were from Vitalriver experimental animal technical co., LTD of Beijing. All animal experiments were approved by the Animal Care and Use Committee of Nanjing Medical University (approval No:14030158) and were performed in accordance with institutional guidelines.

Antibodies

Rabbit polyclonal anti-Arl2 (Cat#: 10232-1-AP) and rabbit anti-Arf5 (Cat#: 20227-1-AP) were purchased from Proteintech Inc. (Chicago, IL, USA). Mouse monoclonal anti-α-tubulin antibody (Cat#:sc-8035) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Human anti-centromere CREST antibody (Cat#: 15–234) was purchased from Antibodies Incorporated (Davis, CA, USA). Cy2-conjugated donkey anti-mouse IgG (Cat#: 715-225-150), Cy2-conjugated donkey anti-rabbit IgG(Cat#: 711-225-152) and Cy3-conjugated donkey anti-human IgG (Cat#: 711-225-152) were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA, USA).

Oocytes collection and culture

Immature oocytes arrested in prophase I (GV oocytes) were obtained from the ovaries of 3-4 week-old ICR female mice. The mice were sacrificed by cervical dislocation and ovaries were isolated and placed in operation medium (Hepes) with 2.5 nM milrinone and 10% FBS (Gibco). Oocytes were released from the ovary by puncturing the follicles with a hypodermic needle. Cumulus cells were washed off the cumulus-oocyte complexes (COC) and every 50 Isolated denuded oocytes were placed in 100 µl droplets of culture medium under mineral oil (Sigma) in plastic dishes (BD). The culture medium was MEM+ (MEM with 0.01 mM EDTA, 0.23 mM Na-pyruvate, 0.2 mM pen / sterep, 3 mg / ml BSA and 20% FBS). Oocytes were cultured at 37.0℃, 5% O_2, 5% CO_2 in humidified atmosphere. Prior to IVM (in vitro maturation), all MEM+

include 2.5 nM milrinone to prevent resumption of meiosis.

Antibody transfection

For antibody transfection, Chariot™ Protein Delivery Reagent (Active motif, Carlsbad, CA, USA) was used. Briefly, Two tubes, one containing 1 µl chariot (1mg / ml in 50% DMSO) in 5 µl sterile water and the other containing 1 µg antibody in PBS (final volume is also 6 µl) were first set up, then solutions from the two tubes were mixed together gently and incubated at room temperature for 30 min to allow the formation of chariot-IgG complex, then the complex solution was added into a 100 µl MEM+ drop containing 50 oocytes. After 12–14 hour treatment, the oocytes was washed to remove the complex-containing MEM+, wait for 1–2 hour and in sequence another two rounds of antibody treatment were exerted to ensure the effectiveness of the antibody inhibition. During the whole treatment, typically 40–44 hour long, 2.5 nM milrinone was always included to prevent resumption of meiosis. Next, oocytes were transfered into milrinone-free MEM+ and cultured for 8 or 16 hour, then subjected to phenotype analysis-related experiments below. Antibodies for transfection have been thoroughly buffer exchanged (over 10^4 dilution of original buffer) into PBS / 50% glycerol to remove antiseptics (usually NaN_3) in the original package.

Immunofluorescence

Oocytes were briefly washed in PBS with 0.05% polyvinylpyrrolidone (PVP), permeated in 0.5% Triton X-100 / PHEM (60 mM PIPES, 25 mM Hepes pH 6.9, 10 mM EGTA, 8 mM MgSO4) for 5 min and washed three times rapidly in PBS / PVP. Next the oocytes were fixed in 3.7% paraformaldehyde (PFA) / PHEM for 20 min, washed three times (10 min each) in PBS / PVP and blocked with blocking buffer (1% BSA / PHEM with 100 mM glycine) at room temperature for 1 hour. Then the oocytes were in sequence incubated at 4℃ overnight with primary antibody diluted in blocking buffer, washed three times (10 min each) in PBS with 0.05% tween-20 (PBST), incubated at room temperature for 45 min with secondary antibody diluted in blocking

buffer (1:750 in all cases), washed three times (10 min each) in PBST. Finally DNA was stained by 10 µg / ml Hochest 33258 and the oocytes were mounted onto a slide with mounting medium (0.5% propgal gallate, 0.1M Tris-Hcl, PH7.4, 88% Glycerol) and covered with a cover glass (0.13–0.17 µm thick). To maintain the dimension of the oocytes, two strips of double-stick tap (90 µm thick) were sticked between the slide and cover glass. Dilution of primary antibody are as follows: anti-Arl2, 1:200; anti-Arl2, 1:200; anti-α-tubulin, 1:500; anti-human centromere, 1:500. The oocytes were examined with an Andor Revolution spining disk confocal workstation (Oxford instruments, Belfast, Northern Ireland).

Data analysis and statistics

All experiments were repeated at least three times, Measurement on confocal Images was done with Image J. Data were presented as x ±Sem. Statistical comparison was done with Student's test. $P<0.05$ was considered to be statistically significant.

RESULTS

A peptide nanoparticle-encapsulated antibody can effectively enter intact mouse oocytes

To test whether a peptide nanoparticle-encapsulated antibody can effectively enter intact mouse oocytes, we used a Rhodamine-conjugated control IgG and compared the cytoplasmic fluorescence of control oocytes, oocytes incubated with Rhodamine-IgG only, and oocytes incubated with peptide nanoparticle-complexed Rhodamine-IgG. We treated the GV oocytes and cultured them till MII. As shown in Figure 1A, the cytoplasmic fluorescence of control oocytes was very low, cytoplasmic fluorescence of oocytes incubated with Rhodamine-IgG only was higher than that of the controls, whereas the cytoplasmic fluorescence of oocytes incubated with peptide nanoparticle-complexed Rhodamine-IgG was significantly higher than that of the former two groups (Fig. 1A and 1B), indicating that peptide nanoparticle-complexed Rhodamine-IgG was effectively delivered into the oocytes. Furthermore, all oocytes from the three groups looked very healthy and developed to MII stage simultaneously, suggesting that the peptide nanoparticle

did not harm the mouse oocytes.

Peptide nanoparticle-mediated antibody transfection can specifically inhibit the target protein

To test whether a peptide nanoparticle-encapsulated antibody can specifically inhibit the target protein while at the same time not affecting others, we selected Arl2 (Arf-like 2) as a target protein and Arf5 (ADP-ribosylation factor 5) as a control protein of the same family. These proteins both belong to the GTP-binding proteins of the Ras superfamily and share high similarity (Fig. 2A). The Arl2 antibody we selected was raised against 2–182 AA of Arl2 (Fig. 2A, blue underlined), whereas the Arf5 antibody was raised against 96–106 AA of Arf5 (Fig. 2A, red underlined). Immunofluorescence showed that both proteins localize within the spindles and share similar localization patterns throughout meiosis (Fig. 2B and 2C). Thus, if Arl2 delivered via peptide nanoparticle became less specific, it could potentially bind to Arf5 as well, in which case Arf5 staining detected by a specific Arf5 antibody would significantly decrease. However, we found no significant difference between the control IgG- and anti-Arl2 antibody-treated groups (Fig.2D). This result indicates that peptide nanoparticle-mediated antibody transfection can inhibit the target protein with very high specificity, i.e., without affecting other members of the same family.

Effective protein inhibition through peptide nanoparticle-mediated antibody transfection can be used in protein function analysis

To test whether peptide nanoparticle-mediated antibody inhibition could be a powerful tool in studies of the functions of unknown proteins, we analyzed the meiotic phenotype after the antibody inhibition of Arl2. Because Arl2 mainly localizes within spindles, we hypothesized that it may function in organizing spindles so that the loss of function could affect spindle integrity and meiosis. Thus, we did a systematic phenotypic analysis on the meiotic spindles at 8 and 16 h of IVM. At 8h, there were significantly more oocytes with clumped chromosomes and without

discernable chromatids and spindle microtubules in the Arl2 inhibition group than in the control group (Fig. 3A and 3B, control vs. Arl2, 0.95% vs. 50.18%), and we called these oocytes as "GV-like" oocytes. There were also significantly fewer oocytes at MI (Fig. 3B, control vs.Arl2, 44.31% vs. 3.07%) in the Arl2 inhibition group than in the control group. Moreover, the spindle length of MI oocytes in the Arl2 inhibition group was significantly shorter than that in the control group (Fig. 3C and 3D, control vs.Arl2, 12.30 μm vs.9.43 μm). At 16 h, there were significantly fewer total MII oocytes (Fig. 3E and 3F, control vs. Arl2, 53.33% vs. 26.19%) in the Arl2 inhibition group than in the control group. Furthermore, all MII oocytes in the Arl2 inhibition group also had uncongressed chromosomes and kinetochores (Fig. 3E, arrow pointed), and we called these oocytes as "Pre-MII oocytes" (Fig. 3F, control vs. Arl2, 44.15% vs. 100%). These results indicate that Arl2 does function in organizing spindles, thereby promoting meiosis progression.

DISCUSSION

To our knowledge, this is the first study showing that through peptide nanoparticle-mediated antibody transfection, antibodies can effectively enter intact oocytes and inhibit specific proteins without affecting other members of the same family. In this method, aChariot peptides form non-covalent bonds with the antibody, stabilizes the antibody, protects it from degradation, and preserves its natural characteristics during the transfection. After delivery, the Chariot peptide-antibody complex dissociates and releases the antibody (Aoshiba&Yokohori& Nagai, 2003; Morris et al., 1999; Morris et al., 2001). We have also successfully established a standardized protocol for the effective delivery of siRNA into mouse oocytes through peptide nanoparticle-mediated siRNA transfection. However, the maximum reduction percentage of a target protein by siRNA is usually only approximately 70%, and if a protein is very abundant, the remaining protein can still be enough to retain its normal function. Therefore, the optimal approach for the knockdown of a highly rich protein is a combination of siRNA knockdown and antibody inhibition. If the protein content is low, antibody inhibition alone may be enough. In summary,

nanoparticle-mediated antibody inhibition is an effective approach to use when studying an unknown protein in mouse oocytes.

Arl2 belongs to the ARF family of small GTP-binding proteins of the Ras superfamily, it interacts with the tubulin-specific chaperone protein known as cofactor D and is involved in the folding of tubulin peptides (Shern et al., 2003). In mitosis, Arl2 is present in centrosomes and regulates tubulin polymerization, thereby affecting cell cycle progression (Zhouet al., 2006; Beghin et al., 2007). However, its role in female meiosis has never been addressed. In the current study, we found that Arl2 localized within spindles and that the inhibition of Arl2 caused substantial spindle defects and significantly delayed meiosis progression. This demonstrates that peptide nanoparticle-mediated antibody inhibition can be used in meiosis studies in mouse oocytes.

In conclusion, for the first time, we have successfully developed peptide nanoparticle-mediated antibody transfection for effective protein inhibition in mouse oocytes. We believe that this new tool will promote further meiosis studies in mouse oocytes.

ACKNOWLEDGEMENTS

We thank Professor Youqiang Su (State key lab of reproductive medicine, Nanjing Medical University) for discussion and advice about this project.

REFERENCES

Mehlmann LM, Jones TL, Jaffe LA. 2002. Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte. *Science* **297(5585):**1343-1345. DOI 10.1126/science.1073978

Yin S, Wang Q, Liu JH, Ai JS, Liang CG, Hou Y, Chen DY, Schatten H, Sun QY. 2006. Bub1 prevents chromosome misalignment and precocious anaphase during mouse oocyte meiosis. *Cell Cycle* **5(18):**2130-2137. DOI 10.4161/cc.5.18.3170

Wang Q, Ai JS, Idowu Ola S, Gu L, Zhang YZ, Chen DY, Sun QY. 2008. The spatial relationship between heterochromatin protein 1 alpha and histone modifications during mouse oocyte meiosis. *Cell Cycle* **7(4):**513-520. DOI 10.4161/cc.7.4.5356

Kondo Y, Fushikida K, Fujieda T, Sakai K, Miyata K, Kato F, Kato M. 2008. Efficient delivery of antibody into living cells using a novel HVJ envelope vector system. J Immunol Methods. *Mar* **20; 332(1-2):**10-17. DOI 10.1016/j.jim.2007.12.008.

Aoshiba K, Yokohori N, Nagai A. 2003. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol* **28(5):**555-562. DOI 10.1165/rcmb.2002-0090OC

Morris MC, Robert-Hebmann V, Chaloin L, Mery J, Heitz F, Devaux C, Goody RS, Divita G. 1999. A new potent HIV-1 reverse transcriptase inhibitor. A synthetic peptide derived from the interface subunit domains. *J Biol Chem* **274(35):**24941-24946. DOI 10.1074/jbc.274.35.24941

Morris MC, Depollier J, Mery J, Heitz F, Divita G. 2001. A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat Biotechnol* **19(12):**1173-1176. DOI 10.1038/nbt1201-1173

Shirai H, Prades C, Vita R, Marcatili P, Popovic B, Xu J, Overington JP, Hirayama K, Soga S, Tsunoyama K, Clark D, Lefranc MP, Ikeda K. 2014. Antibody informatics for drug discovery. *Biochim Biophys Acta* **1844(11):**2002-2015. DOI 10.1016/j.bbapap.

Shern JF, Sharer JD, Pallas DC, Bartolini F, Cowan NJ, Reed MS, Pohl J, Kahn RA. 2003. Cytosolic Arl2 is complexed with cofactor D and protein phosphatase 2A. *J Biol Chem* **278(42):**40829-40836. DOI 10.1074/jbc.M308678200

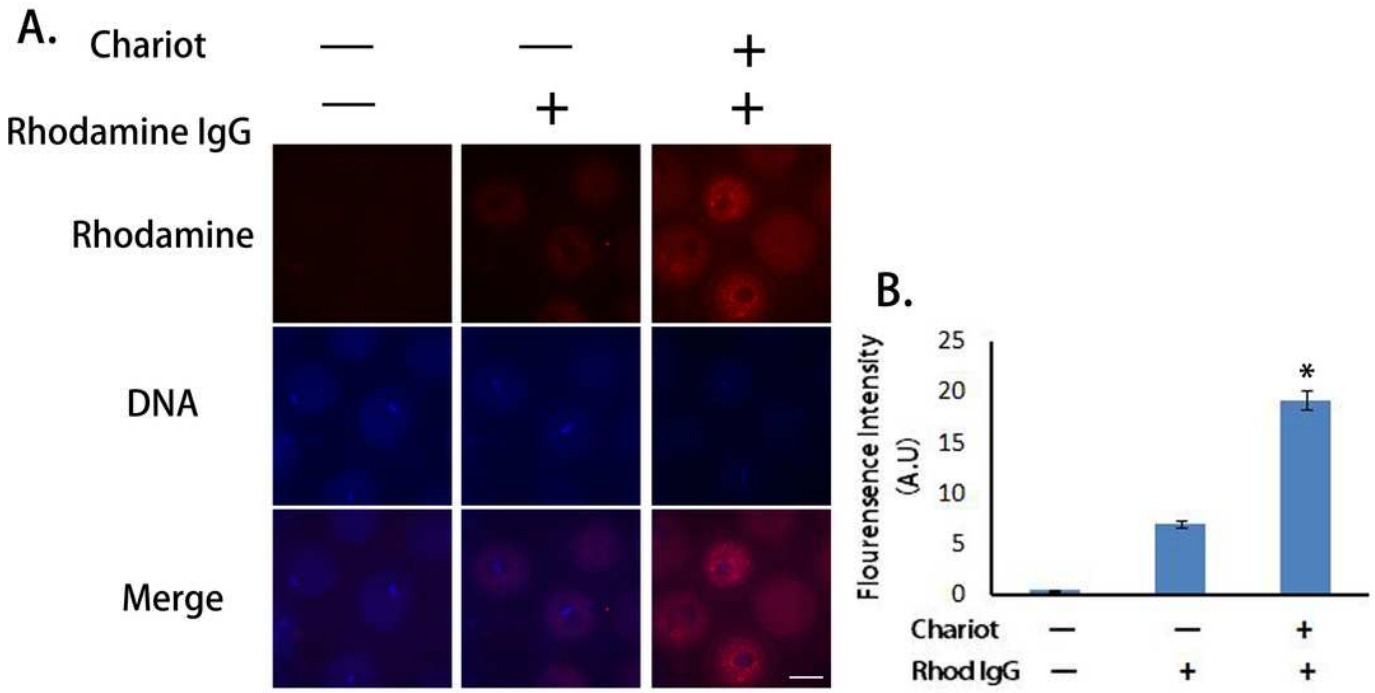
Zhou C, Cunningham L, Marcus AI, Li Y, Kahn RA. 2006. Arl2 and Arl3 regulate different microtubule-dependent processes. *Mol Biol Cell* **17(5):**2476-2487. DOI 10.1091/mbc.E05-10-0929

Beghin A, Honore S, Messana C, Matera EL, Aim J, Burlinchon S, Braguer D, Dumontet C. 2007. ADP ribosylation factor like 2 (Arl2) protein influences microtubule dynamics in breast cancer cells. *Exp Cell Res* **313(3):**473-485. DOI 10.1016/j.yexcr.2006.10.024

1

Peptide nanoparticle-encapsulated antibody can effectively enter mouse oocytes

A, Fluorescence comparison between three different groups. Left, control oocytes without any treatment; middle, oocytes were incubated with rhodamine-conjugated rabbit IgG; right, oocytes were incubated with peptide nanoparticle-encapsulated rhodamine-conjugated rabbit IgG. B, Quantification of rhodamine fluorescence of three groups in A. Significant comparison (p□ 0.05) were aster (*) marked. Scale bar, 20 μm.



2

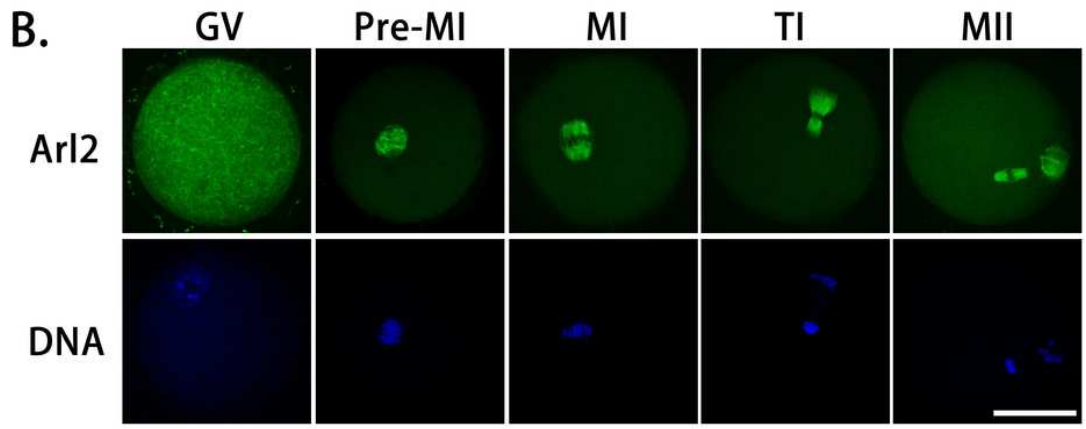
Peptide nanoparticle-mediated antibody transfection can specifically inhibit target protein in mouse oocytes

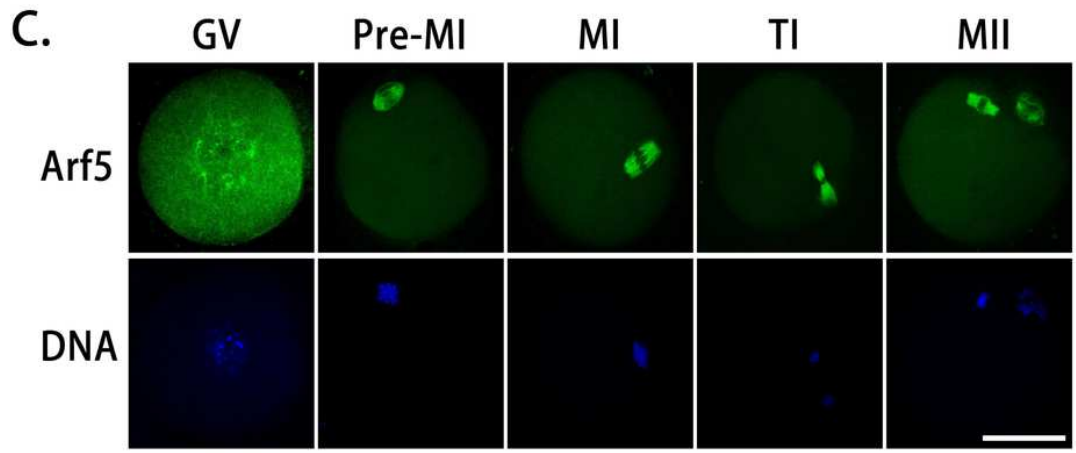
A, Protein sequence alignment of Arl2 and Arf5. Blue-underlined Arl2 sequence (2–182 AA) are antigen region for anti-Arl2 antibody, red-underlined Arf5 sequence (96–106 AA) are antigen region for anti-Arf5 antibody. B, Arl2 immunolocalization in mouse oocytes at each meiotic stage. From left to right, GV, Pre-MI, MI, TI, MII. C, Arf5 immunolocalization in mouse oocytes at each meiotic stage. From left to right, GV, Pre-MI, MI, TI, MII. D, Immunostaining of Arf5 at MI stage in control IgG (left) or anti-Arl2 antibody (right) transfection group . E, Quantification of Arf5 fluorescence at MI stage in control IgG (left) or anti-Arl2 antibody (right) transfection oocytes. Scale bar, 20 μm.

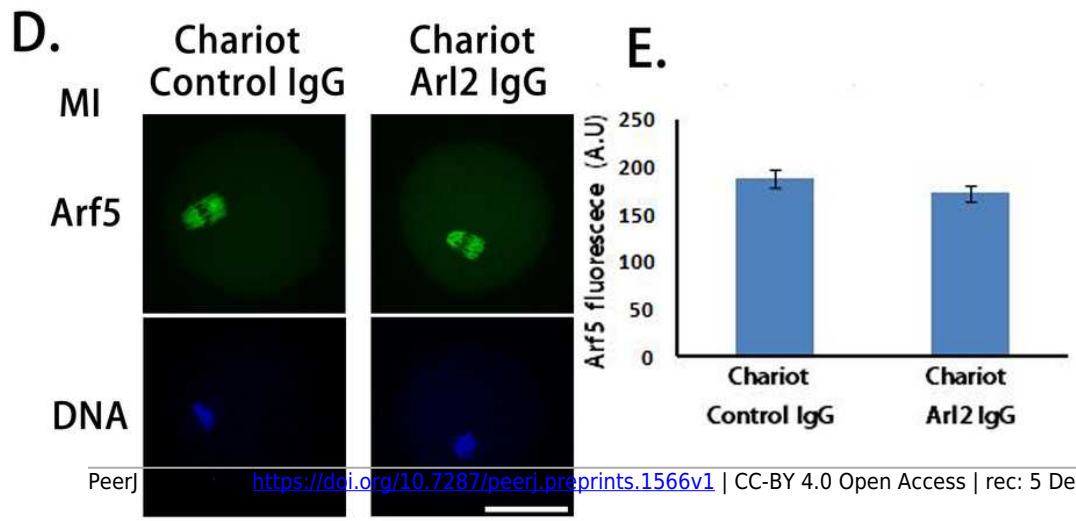
PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1566v1 | CC-BY 4.0 Open Access | rec: 5 Dec 2015, publ: 5 Dec 2015

A.

```
Ar12    MGLLT-ILKKMKQKERELRLLMLGLDNAGKTTILKKFNGEDVDTISPTLGFNIKTLEHRG  59
Arf5    MGLTVSALFSRIFGKKQMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIGFNVETVEYKN  60
        *** .  * .     :::::*:**:*** ******* *:: :: * **:***::*:*::

Ar12    FKLNIWDVGGQKSLRSYWRNYFESTDGLIWVVDSADRQRMQDCQRELQSLLVEERLAGAT  119
Arf5    ICFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERVQESADELQKMLQEDELRDAV  120
        : :.:******..:*  **.**:.*:***:**** **:*:*:.  ***.:* *:.*  *.

Ar12    LLIFANKQDLPGALSCNAIQEALELDSIRSHHWRIQGCSAVTGEDLLPGIDWLLDDISSR  179
Arf5    LLVFANKQDMPNAMPVSELTDKLGLQHLRSRTWYVQATCATQGTGLYDGLDWLSHELSKR  180
        **:******:* *:  . : : * *: :**: * :*. .*. *  *  *:*** .::*.*

Ar12    VFTAD 184
Arf5    ----- 180
```







3

Effective protein inhibition through peptide nanoparticle-mediated antibody transfection can be used in protein function analysis in mouse oocytes

A, B, At 8 hour, there were significantly more GV-like and less MI oocytes in anti-Arl2 antibody transfection group than in control. B, Percentage of oocytes at different stages at 8 hour of IVM. C, MI spindle in anti-Arl2 antibody transfection group was significantly shorter than in control. D, Quantification of MI spindle length and width in control or anti-Arl2 antibody transfection group. E and F, At 16 hour, the percentage of total MII oocytes in anti-Arl2 antibody transfection group was significantly lower than in control. And there were significantly more Pre-MI oocytes in anti-Arl2 antibody transfection group than in control. Significant comparison (p 0.05) were aster (*) marked. Scale bar, 20 μm.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1566v1 | CC-BY 4.0 Open Access | rec: 5 Dec 2015, publ: 5 Dec 2015